PATENT
VAN67 P328A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	: 1635
Examiner	: Louis V. Wollenberger
Applicant	: Nariyoshi Shinomiya et al.
Appln. No.	: 10/599,327
Filing Date	: March 12, 2007
Confirmation No.	: 7013
For	: *c-met* siRNA ADENOVIRUS VECTORS INHIBIT CANCER CELL GROWTH, INVASION AND TUMORIGENICITY

DECLARATION UNDER 37 C.F.R. § 1.131

We, the undersigned, do hereby declare as follows:

1. We are the co-inventors of the claims of the above-identified patent application.

2. The invention as defined in claims 1-12, 14-17, and 38 was conceived of and actually reduced to practice prior to January 6, 2003. The invention as defined in claims 13, 18-20, and 48-50 was conceived of and actually reduced to practice prior to July 7, 2003.

3. Evidence of our conception and reduction to practice of the invention as defined in claims 1-20, 38, and 48-50 is provided in the form of experimental data from the laboratory notebook of Nariyoshi Shinomiya, one of the named inventors (attached hereto as Exhibit A1-A10). More specifically, these laboratory notebooks show our development of an RNAi molecule directed to c-met:

a) in the cancer cell lines DU-145, SK-LMS-1, DA3, and M114 (Exhibit A1);

b) using siRNA expression vector pSilencer 1.0-U6 for human c-met (Exhibit A2 and A4);

c) targeting human c-met sequence 221, the target of SEQ ID No. 15 (Exhibit A3);

d) using pShuttle vector (Exhibit A5);

e) using an Ad5 viral vector (Exhibit A6 and A9);

f) using a stable transformant (Exhibit A7 and A8); and

g) in DBTRG glioblastoma cells (Exhibit A10).

4. The documents attached as Exhibit A1-A10 were prepared contemporaneously with our conception and reduction to practice.

5. The acts referred to in the preceding paragraphs occurred in the United States.

6. The undersigned hereby declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Sections 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

September 30, 2009
Date

George F. Vande Woude

N. Shinomiya

September 26, 2009
Date

Nariyoshi Shinomiya

< Cell culture from the stock >

① DU-145 ~~~~ RPMI 1640 + 5% FBS
(−160°C stock = 1/11/2007) (+ 1% pen/strep)

② SK-LMS-1 ~~~~ DMEM + 10% FBS
(passage 2) ATCC (#11965-092) (+ 1% pen/strep)

③ DA3 ~~~~ DMEM + 10% Calf Serum
(−160°C stock = 3/23/01) or Fetal Bovine Serum
(+ 1% pen/strep)

④ M114 ~~~~ DMEM + 10% Calf Serum
(−160°C stock = 10-26-01) or Fetal Bovine Serum
(+ 1% pen/strep)

Culture media & Cell preservation media

* 5% FBS-RPMI 1640 (cat No.: 11875-093
Lot No.: 1140467
FBS 15ml
P/S 3ml
RPMI 282ml

* 10% FBS-DMEM (Cat No.: 11965-092
Lot No.: 1430062
FBS 30ml
P/S 3ml
DMEM 257ml

* Cell preservation media
DMSO 2ml
FBS 20ml
RPMI 78ml
→ DMSO 7ml
FBS 20ml
DMEM 73ml

EXHIBIT A1

< Transformation of pSilencer™ 1.0-U6 vector into TOP10 competent cells >

TOP10 → from -80°C freezer

↓

50µl → eppendorf tube on ice

↓

pSilencer 1.0-U6 vector 0.2µl (= 150ng)

↓

25 min on ice

↓

30 sec at 42°C

↓

add 250µl SOC

↓

Shaking incubation (37°C, 250 rpm)

↓

plate on amp LB plate (50µl, 100µl)

pSilencer 1.0-U6

< continued >

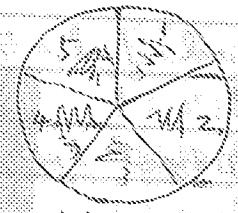
100µl → colonies are too many (can not be picked up)

50µl → ~ 200 colonies

↓

5 colonies are picked up

↓



duplicated plate ① → plasmid purification

② → Stock of cells

LB-amp

↓

37°C

EXHIBIT A2

<Transformation mouse siRNA – part 4>

① LB amp Agar

			pick up	
#57	8 colonies	⟶	(1, 2, 3)	
#60	1 colony	⟶	(1)	⟹ LB amp medium 2ml shaking incubation (9:05 am ~)
#110	1 "	⟶	(1)	
#120	0 "	⟶	X	
#178	5 colonies	⟶	(1, 2, 3)	

* Transformation efficiency was not very good!

~~Since human siRNA oligo transformation obtained a good result, maybe there is a problem in the conc. of siRNA oligos.~~

⇓

~~Try again by using higher conc of siRNA oligos.~~

↳ # 60, # 110, # 120 only

② Ligation again

mU6 pro BbsI / XbaI digest & gel purified 0.5µl
X 100 oligo 2µl
5 x Ligase buffer 2µl
water 5µl
T4 DNA Ligase 0.5µl
Total 10µl

⇓

10:25 am ⟶ Incubate at RT for 30 min ⟶ Add EDTA / five fold diluted ⇓ Transformation in the same way as previously ↘ LB amp

[redacted] [redacted] [redacted]

(~ 7.5 hr incubation) 37°C, 250 strokes

4:25 pm ⟶ plasmid extraction using QIAGEN miniprep kit

⇓

Analysis by electrophoresis ↳ (X) page 22b

EXHIBIT A3
Page 1 of 4

< Confirmation of the extracted plasmids >

① Digestion of plasmids with restriction enzymes

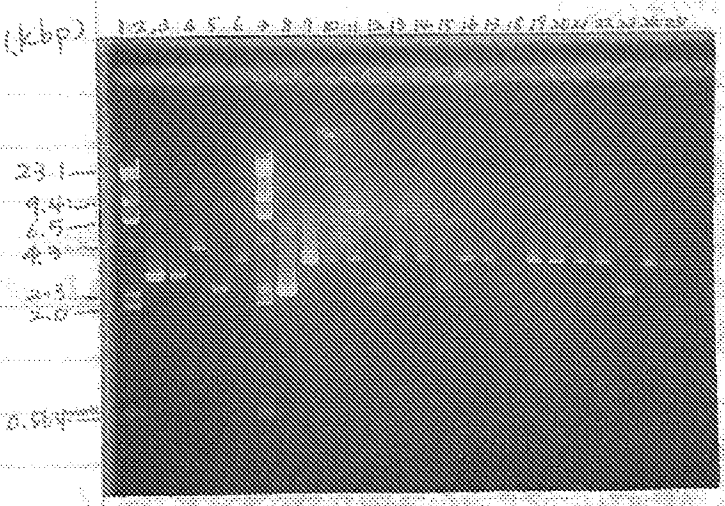
[#57, #178 → XbaI (Buffer 2)

#16-1,2,3,4 #62-1,2,3,4 #76-1,2,3,4 #178-1,2,3,4
→ HindIII (Buffer 2)]

* 1μl each plasmid was digested at 37°C for 1hr

② Electrophoresis (1% agarose gel, GENE Mate 150 V for 1.5hr)

→ gel stain — 150ml water + 30μl EtBr (10mg/ml)



1 — λ HindIII
2 — mU6 (circular)
3 — #57
4 — #57/XbaI
5 — #178
⑥ — #178/XbaI (#178-1)
7 — λ HindIII
8 — pSilencer (circular)
9 — pSilencer/HindIII (linear)
10 — #16-1
11 — 〃 2
⑫ — 〃 3
13 — 〃 4
14 — #62-1
⑮ — 〃 2
16 — 〃 3
17 — 〃 4
⑱ — #76-1
19 — 〃 2
20 — 〃 3
21 — 〃 4
22 — #221-1
㉓ — 〃 2
24 — 〃 3
25 — 〃 4

(10–25: HindIII digest)

(A) Mouse siRNA
XbaI digest → 3.4kb is OK

[(mU6 = 4143 nt)
mU6 Bbs1/XbaI ≒ 3.4kb
mU6 insert ≒ 3.4kb]

(B) human siRNA
after HindIII digestion
still circular is OK
(≒3.3Kb)

○ → to be sequenced!

③ Sequencing

- #128 (#128-0) → mouse siRNA for c-Met (M13 R2 primer)
- #16 (#16-3)
- #62 (#62-2)
- #26 (#26-1)
- #221 (#221-2)

→ human siRNA for c-Met (T3 primer (A))

Template plasmid ___ μl (e.g. 2μg)
Primer 1μl (3.2 pmol)
↳ sequencing

* Primers

M13 R2 - [illegible] pmol → dissolved in 268μl of water (≒ 300 pmol/μl)

T3 (A) - 256.0 pmol → dissolved in 269μl of water (≒ 300 pmol/μl)

→ store at -20°C

↓

Then 10 fold dilution with water
primer : water = 10 μl : 90 μl → use 1μl for sequencing

→ Stok (250pmol/μl) −20°C

siRNA hairpin template sequences for human c-met RNAi

A. Criteria of Sequence Selection

1. 21mer that start with AA
2. GC content between 45-55%
3. No more than three consecutive T or G nucleotides can be present anywhere in the hairpin template sequences
4. The targeted region is selected from a given cDNA sequence beginning 50 to100 nt downstream of the start codon. (5' or 3' UTRs and regions nearby the start codon are avoided, as these may be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNP or RISC endonuclease complex.)
5. Blasted selected sequences against Genbank
6. Nucleotide cross-match (no more than 17, much less would be better)
7. Start with AAG (for cloning into pSilencer 1.0-U6 vector system; RNA transcription begin with G, but according to the company's information a +1 G is not required.)
8. A pair of template sequences is as follows:

	Sense	Loop	Antisense	
Template 1:	5'-N(19)-	TTCAAGAGA-	N(19)-TTTTTT-3'	(53mer)
Template 2:	3'-CCGG-N(19)-	AAGTTCTCT-	N(19)-AAAAAATTAA-5'	(61mer)
	ApaI		EcoRI	

Target sequence 58: AAGACCTTCAGAAGGTTGCTG
Blast search (in new window)
Position in gene sequence: 415
GC content: 47.6%
siRNA Sense strand: GACCTTCAGAAGGTTGCTGTT
siRNA Antisense strand: CAGCAACCTTCTGAAGGTCTT
#16-1: 5'-GACCTTCAGAAGGTTGCTG-TTCAAGAGA-CAGCAACCTTCTGAAGGTC-TTTTTT-3'
#16-2: 3'-CCGG-CTGGAAGTCTTCCAACGAC-AAGTTCTCT-GTCGTTGGAAGACTTCCAG-AAAAAATTAA-5'
#16-2: 5'-AATTAAAAAA-GACCTTCAGAAGGTTGCTG-TCTCTTGAA-CAGCAACCTTCTGAAGGTC-GGCC-3'

Target sequence 62: AAGCCAGATTCTGCCGAACCA
Blast search (in new window)
Position in gene sequence: 1236
GC content: 52.4%
siRNA Sense strand: GCCAGATTCTGCCGAACCATT
siRNA Antisense strand: TGGTTCGGCAGAATCTGGCTT
#62-1: 5'-GCCAGATTCTGCCGAACCA-TTCAAGAGA-TGGTTCGGCAGAATCTGGC-TTTTTT-3'
#62-2: 3'-CCGG-CGGTCTAAGACGGCTTGGT-AAGTTCTCT-ACCAAGCCGTCTTAGACCG-AAAAAATTAA-5'
#62-2: 5'-AATTAAAAAA-GCCAGATTCTGCCGAACCA-TCTCTTGAA-TGGTTCGGCAGAATCTGGC- GGCC-3'

Target sequence 76: AAGCGCGCCGTGATGAATATC
Blast search (in new window)
Position in gene sequence: 1417
GC content: 52.4%
siRNA Sense strand: GCGCGCCGTGATGAATATCTT
siRNA Antisense strand: GATATTCATCACGGCGCGCTT
#76-1: 5'-GCGCGCCGTGATGAATATC-TTCAAGAGA-GATATTCATCACGGCGCGC-TTTTTT-3'
#76-2: 3'-CCGG-CGCGCGGCACTACTTATAG-AAGTTCTCT-CTATAAGTAGTGCCGCGCG-AAAAAATTAA-5'
#76-2: 5'-AATTAAAAAA-GCGCGCCGTGATGAATATC-TCTCTTGAA-GATATTCATCACGGCGCGC- GGCC-3'

Target sequence 221: AAGTGCAGTATCCTCTGACAG
Blast search (in new window)
Position in gene sequence: 3310
GC content: 47.6%
siRNA Sense strand: GTGCAGTATCCTCTGACAGTT
siRNA Antisense strand: CTGTCAGAGGATACTGCACTT
#221-1: 5'-GTGCAGTATCCTCTGACAG-TTCAAGAGA-CTGTCAGAGGATACTGCAC-TTTTTT-3'
#221-2: 3'-CCGG-CACGTCATAGGAGACTGTC-AAGTTCTCT-GACAGTCTCCTATGACGTG-AAAAAATTAA-5'
#221-2: 5'-AATTAAAAAA-GTGCAGTATCCTCTGACAG-TCTCTTGAA-CTGTCAGAGGATACTGCAC- GGCC-3'

< Sequence confirmation of siRNA plasmid >

Expression plasmids for c-met RNAi (final clones)

1. Mouse siRNA (host plasmid = mU6pro)

Target sequence No.	Position in gene sequence	Ligation of synthesized oligos & transformation	Sequence confirmation of the inserted oligos	Final clone #s
#57	950	finished	confirmed	#57-1, #57-2, #57-3
#60	988	finished	confirmed	#60-1, #60-4
#110	1839	finished	confirmed	#110-1, #110-2, #110-3
#120	1977	Very low transformation efficiency	-	-
#178	2762	finished	confirmed	#178-0, #178-1, #178-2

2. Human siRNA (host plasmid = pSilencer)

Target sequence No.	Position in gene sequence	Ligation of synthesized oligos & transformation	Sequence confirmation of the inserted oligos	Plasmid amplification for transfection
#16	415	finished	confirmed	#16-3
#62	1236	finished	confirmed	#62-2
#76	1417	finished	Wrong sequence	-
#221	3310	finished	confirmed	#221-6

#red, #blue : plasmid clones were amplified and used for transfection
Control plasmid: mU6pro = mU6#1, pSilencer = pSil#1

< plasmid amplification ~ #110-1 #221-6 #mU6#1 pSil#1 >

1st 1~2ml → shaking incubation
LBamp
#110-1
#221-6] → from LB plate
mU6#1
pSil#1] → from -80°C stock

2nd 100ml overnight shaking incubation !!

EXHIBIT A4

< Large Scale Plasmid Amplification
— Murine c-Met siRNA expression plasmids >

(1) Yesterday → separation culture from -80°C stock
↓
(2) Today → pick up one colony & shaking culture
in 2ml LB-Amp
(100μg/ml)
[9:00am ~]

Ligation reaction (brief protocol)

a. Stick-ends (Cohesive Ends) Ligation

1. To an eppendorf tube add the following:

5x ligase reaction buffer	4 μL
vector DNA	3-30 fmol (2.5-25 ng)
insert DNA	9-90 fmol (7.5-75 ng)
(total DNA	0.01-0.1 μg)
autoclaved distilled water	to 19 μL
T4 DNA Ligase	1 unit (in 1 μL)
Final volume	20 μL

2. Mix gently. Centrifuge to bring the contents to the bottom of the tube.
3. Incubate at 23°C to 26°C for at least 5 min (30 min would be better).
4. Add 1 μL of 0.5 M EDTA to inactivate the enzyme.
5. Store the reaction at 4°C.
6. Dilute an aliquot of the ligation reaction five-fold in autoclaved distilled water and use it to transform competent cells.

b. Blunt ends Ligation

1. To an eppendorf tube add the following:

5x ligase reaction buffer	4 μL
vector DNA	15-60 fmol (25-250 ng)
insert DNA	45-180 fmol (75-750 ng)
(total DNA	0.1-1.0 μg)
autoclaved distilled water	to 19 μL
T4 DNA Ligase	1 unit (in 1 μL)
Final volume	20 μL

2. Mix gently. Centrifuge to bring the contents to the bottom of the tube.
3. Incubate at 14°C for 16-24 hr.
4. Add 1 μL of 0.5 M EDTA to inactivate the enzyme.
5. Store the reaction at 4°C.
6. Dilute an aliquot of the ligation reaction five-fold in autoclaved distilled water and use it to transform competent cells.

Design (restriction enzyme digestion & ligation)

a. **Murine c-met siRNA**
   pShuttle (vector): XbaI/HindIII double digestion --> gel purification
   mU6pro (insert): HindIII/XbaI double digestion --> gel purification

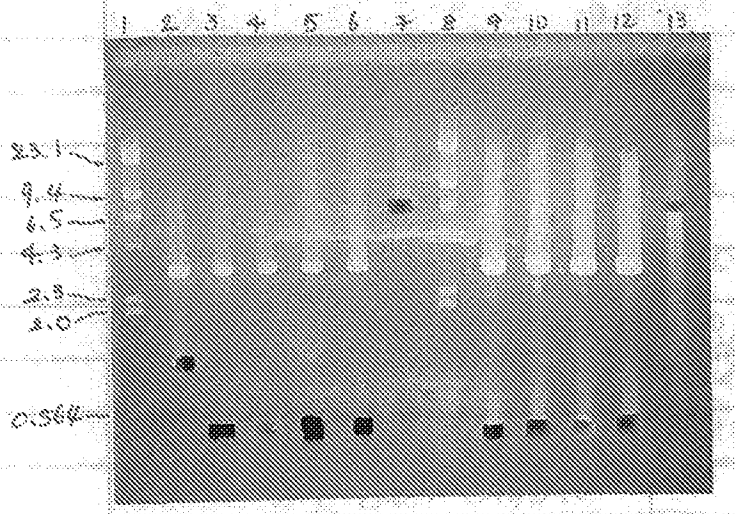
   XbaI = REact 2, 37°C, 1 hr
   HindIII = REact 2, 37°C, 1 hr

   *stick ends ligation (direction of the insert is reverse)

b. **Human c-met siRNA**
   pShuttle (vector): KpnI digestion --> phenol extraction/ethanol precipitation
   EcoRV digestion --> gel purification
   pSilencer (insert): KpnI/SmaI double digestion (30°C then 37°C) --> gel purification

   KpnI = REact 4, 37°C, 1 hr
   SmaI = Gene Choice buffer 4 (=REact 4), 30°C, 1 hr
   EcoRV = Gene Choice buffer 2 (=REact 2), 37°C, 1 hr

   *blunt ends ligation (the same direction)



1 — λ HindIII → ~1.2 kbp
2 — mU6
3 — 57-1
4 — 60-1
5 — 110-1
6 — 178-1
} HindIII/XbaI double digest ~ 6.6 kbp
7 — pShuttle
8 — λ HindIII
9 — p52
10 — 16-3
11 — 62-2
12 — 224-6
} KpnI/SmaI double digest (~ 4.6 kbp)
13 — pShuttle – KpnI/EcoRV digest (~6.6 kbp)

desired bands were recognized !!

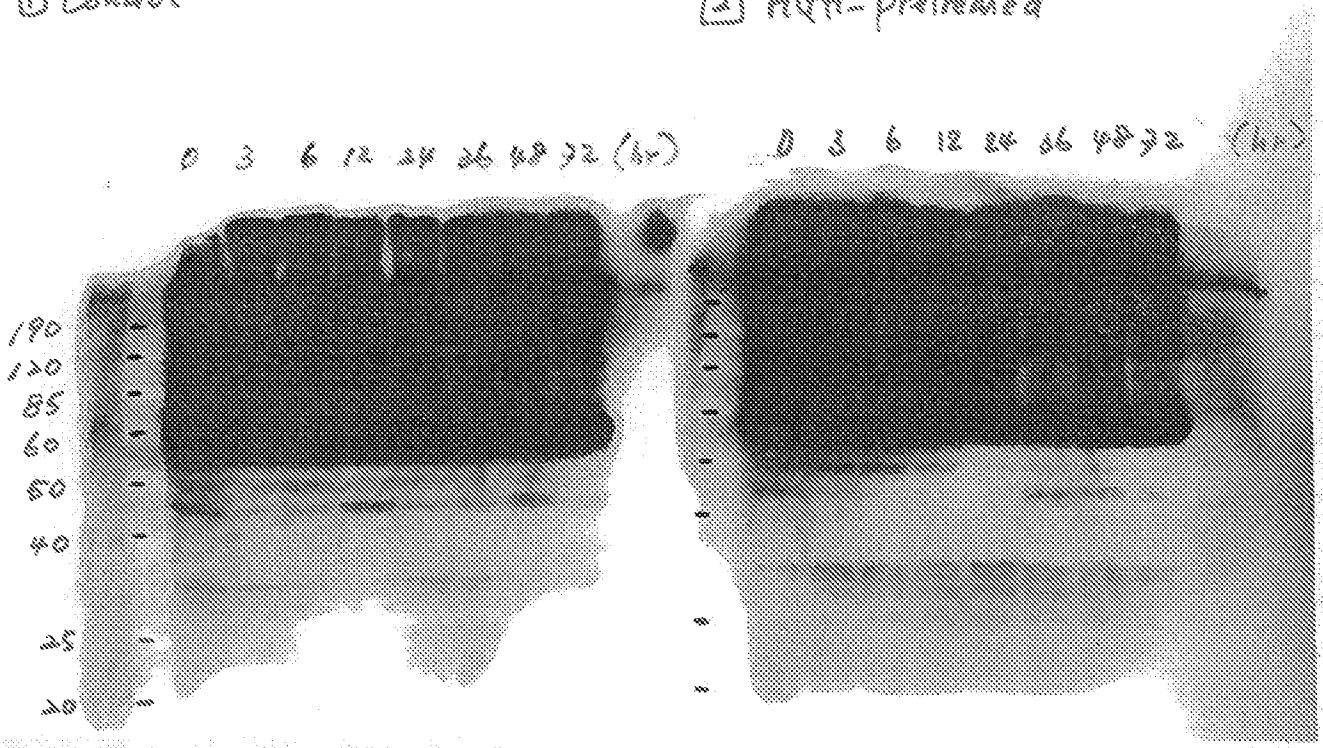
gel purification

elute with H_2O (final vol = 30 µl) (store -20°C until use) → Tomorrow Ligation Reaction

< Western blot ~ Results >

① Control

③ HQR-pretreated



* 50~190 kDa → phosphorylation bands are too strong

↓

[New time load 50μg protein
[Separate with 8% gel

< Adenovirus purification using Virapur >

	OD 260	280	260/280	titre (cfu/ml)
pAd ②	0.161	0.135	1.195	1.8×10^{12}
pAd ③	0.186	0.125	1.495	2.0×10^{12}

pAd ②]
pAd ③] → harvest tomorrow

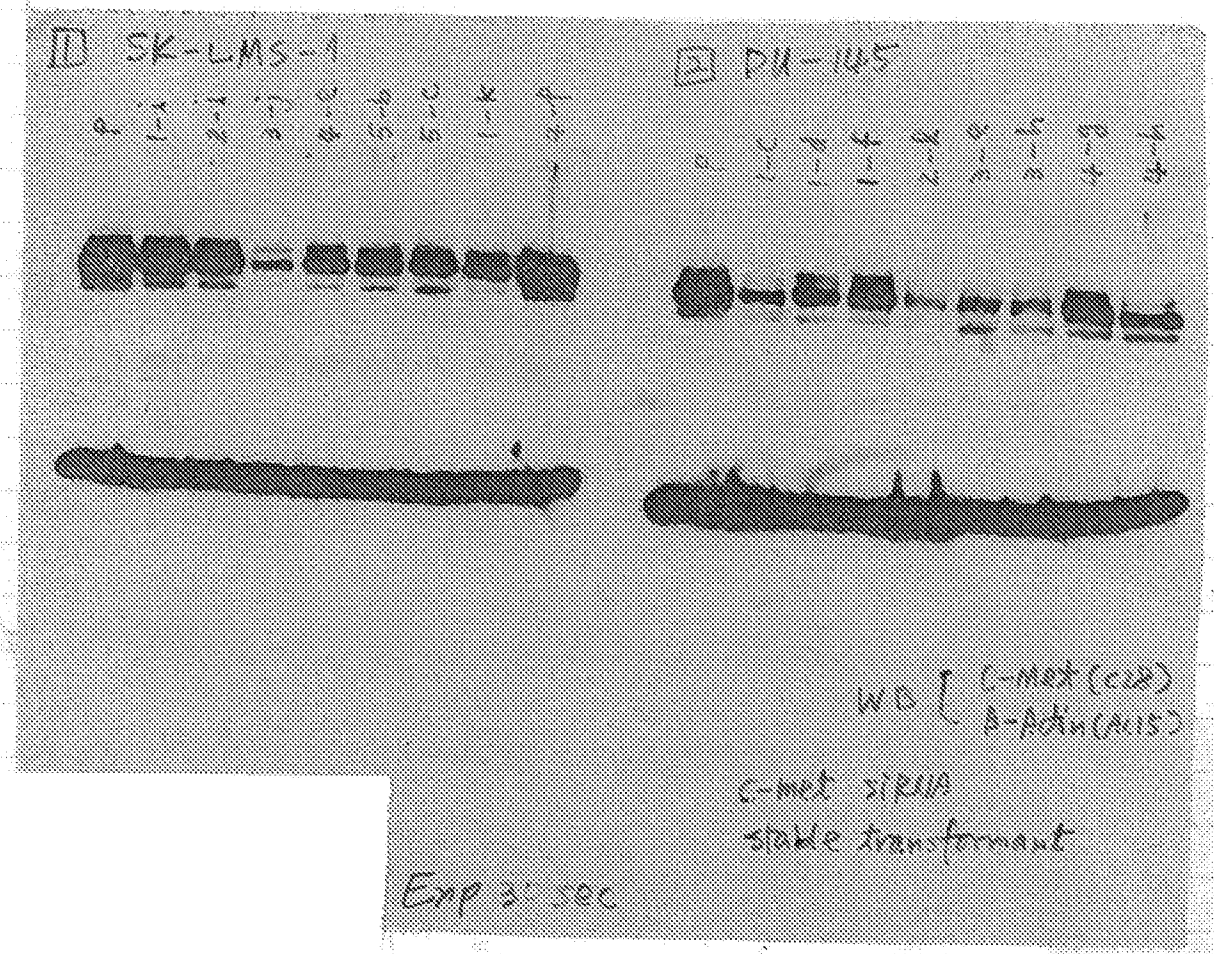
pAd ② , ③
300μl each × 9
+ 1 each
↘ -80°C

< Western blot ~ siRNA transfectant
SK-LMS-1 & DU-145 >

primary Ab
(c-Met (C28) 1:2000
(β-Actin (AC15) 1:5000

2nd day Ab
(α-rabbit 1:1000
(α-Mouse 1:5000

@ protein
20μg was Loaded in each lane
(both SK and DU)



c-Met Downregulation is also observed in DU-145 cells.

< Histology of the remnant livers >

Serial number	Sample name	Serial number	Sample name
GVW-4001	C 12h-1	GVW-4016	H 12h-1
4002	C 12h-2	4017	H 12h-2
4003	C 12h-3	4018	H 12h-3
4004	C 24h-1	4019	H 24h-1
4005	C 24h-2	4020	H 24h-2
4006	C 24h-3	4021	H 24h-3
4007	C 36h-1	4022	H 36h-1
4008	C 36h-2	4023	H 36h-2
4009	C 36h-3	4024	H 36h-3
4010	C 48h-1	4025	H 48h-1
4011	C 48h-2	4026	H 48h-2
4012	C 48h-3	4027	H 48h-3
4013	C 72h-1	4028	H 72h-1
4014	C 72h-2	4029	H 72h-2
4015	C 72h-3	4030	H 72h-3

Difference in the mitotic indices ?

4001 ~ normal, hard to find mitotic cells
4002 ~ small vacuoles ⊕, "
4003 ~ bleeding inside the liver, hyalin-like changes
4004 ~ many vacuoles ⊕⊕
4005 ~ small vacuoles ⊕ (around the vein, no vacuoles)
4006 ~ "
4007 ~ vacuoles ⊕
4008 ~ small vacuoles ⊕
4009 ~ small vacuoles ⊕, normal
4010 ~ small vacuoles ⊕/mitotic cells occasionally
4011 ~ vacuoles ⊕/mitotic cells, occasionally
4012 ~ " / "
4013 ~ mitotic cells 1~3/HPF
4014 ~ "
4015 ~ "

4016 ~ many bleeding sites
4017 ~ small vacuoles ⊕
4018 ~ " ⊕
4019 ~ vacuoles ⊕
4020 ~ " ⊕⊕
4021 ~ " ⊕
4022 ~ vacuoles ⊕ depends on the portion, Mitosis occasionally
4023 ~ vacuoles ⊕, Mitosis occasionally
4024 ~ vacuoles ⊕⊕ hyaline necrosis, inflammation?
4025 ~ vacuoles ⊕ Mitosis 2~5/HPF
4026 ~ mitosis 2~3 /HPF
4027 ~ "
4028 ~ Mitosis 2~3/HPF
4029 ~ "
4030 ~ "

EXHIBIT A8

< Western blot ~ siRNA stable transformant >
Expression of EGFR

① SK-LMS-1

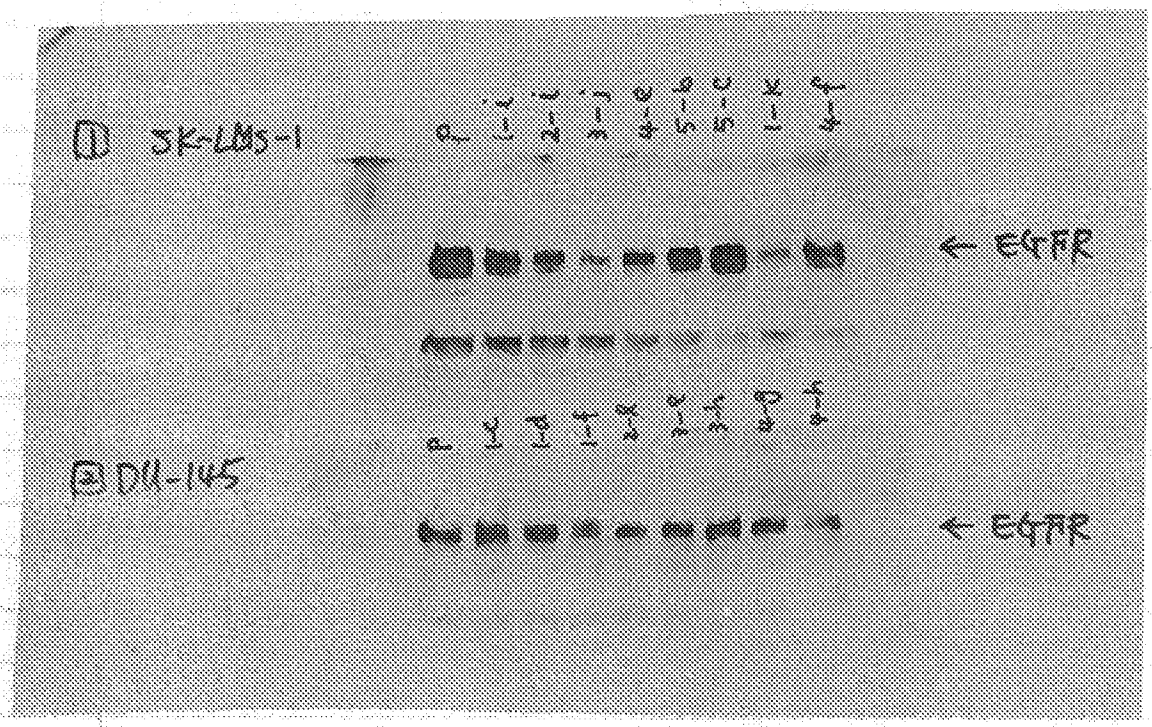
x, M, ⓟ, 1-i, 2-i, 3-j, 4-e, 5-b, 5-c, 1-k, e-f, x ← 20μg protein each loaded

② DU-145

x, M, ⓟ, 1-c, 1-d, 1-f, 2-l, 3-e, 3-h, 4-g, 4-h, x

1st Ab : α-EGFR 1:1000
2nd Ab : α-Rabbit 1:2000

Results ~



② { in SK-LMS-1 → expression of EGFR varies & also correlates well with proliferative activity
in DU-145 → no remarkable changes in the EGFR levels

< Large scale virus production ~ pad ① >

T/25 × 5 flasks

↓

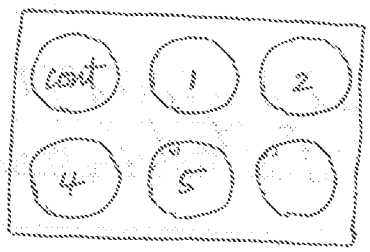
Virapur kit

OD260	OD280	260/280
0.083	0.056	1.55

* cpu/ml = 0.083 × 50 × 1.1 × 10^{12} ≒ $\underline{4.8 \times 10^{12}}$ (cpu/ml)

500μl each × 8 tubes + 1 additional tube → −80°C stored

< Cell seeding activity ~ 293 cells >



10ng/ml HGF was added to each well
(1:30 a.m. ~ ≒ 04)

< Large scale virus production ~ pad ⑤ >

T/25 × 5 flasks ~ almost all cells were detached from the bottom and floated as single cells.

↓

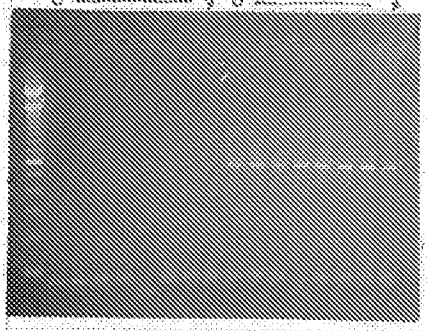
Virapur kit

OD260	OD280	260/280 ratio
0.041	0.029	1.39

particle number = 0.041 × 50 × 1.1 × 10^{12} ≒ $\underline{2.3 \times 10^{12}}$ (cpu/ml)

50μl each × 8 tubes + 1 additional tube → −80°C stock
(>1 ml)

< RT-PCR results >

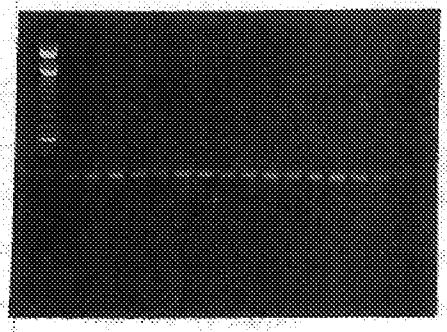


← hHGF (539bp)

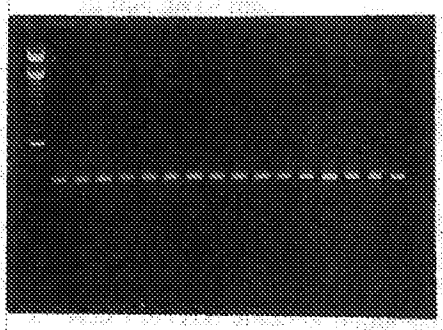
* after 3/3 pH

0	1	2	3	4	5	6	7
0h	3h	6h	12h	24h	36h	48h	72h

↑ no-hHGF expression in sid control mice.



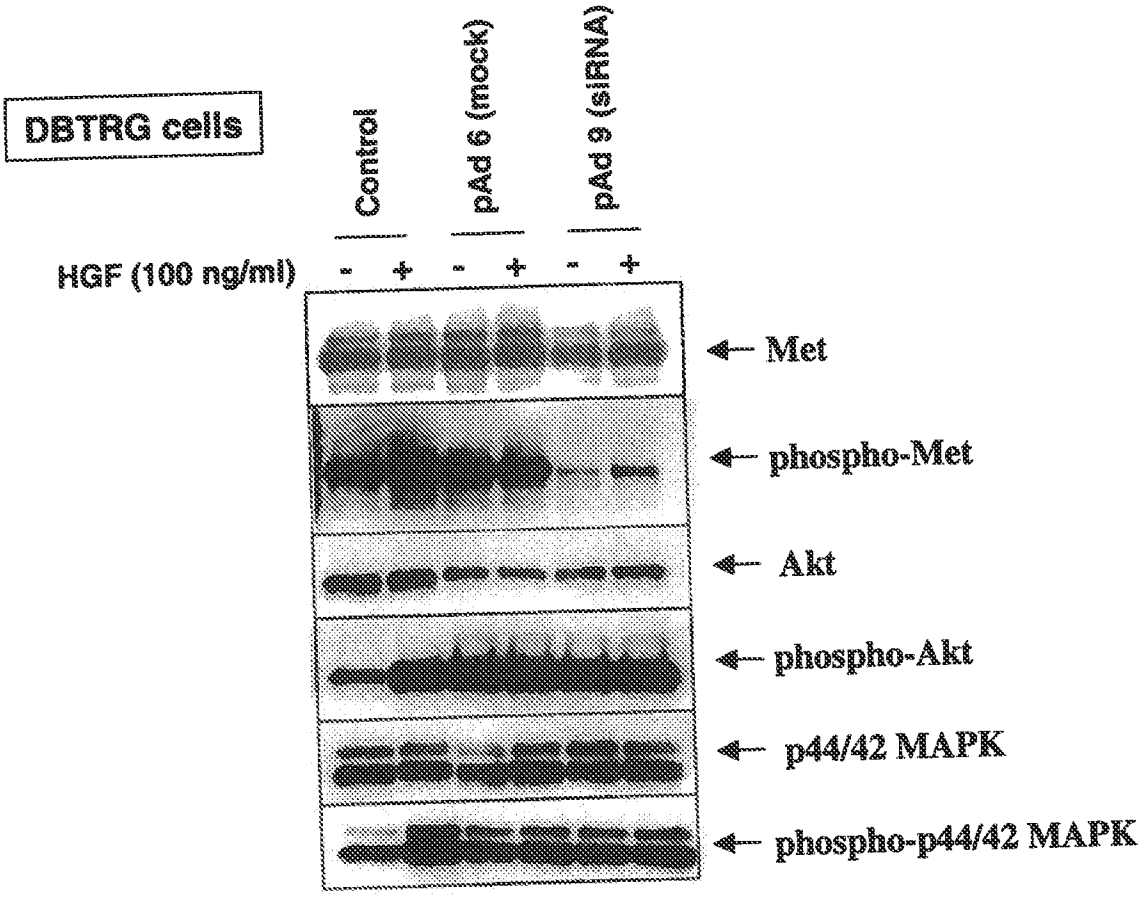
← mHGF (365bp) → no remarkable changes in terms of time course



← m β-actin (376bp)

< DBTRG cells siRNA adv. infect ⇒ HGF stimulation >

siRNA suppresses Met phosphorylation



- Met is down regulated in pAd⑨ infected cells
- Met phosphorylation is also significantly suppressed in pAd⑨ infected cells
- But regarding to the Akt-phosphorylation & p44/42 MAPK phosphorylation strong phosphorylation bands were observed from the beginning (before HGF stimulation)